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NITRIC OXIDE MODULATES CARDIAC PERFORMANCE IN THE HEART OF *ANGUILLA ANGUILLA*

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Summary

Nothing is known about the effects of nitric oxide (NO) on cardiac performance in fish. Using an *in vitro* working heart preparation that generates physiological values of output pressure, cardiac output and ventricular work and power, we assessed the effects of NO on the cardiac performance of the eel *Anguilla anguilla*. We examined basal cardiac performance (at constant preload, afterload and heart rate), the effects of cholinergic stimulation and the Frank–Starling response (preload-induced increases in cardiac output at constant afterload and heart rate). The NO synthase (NOS) inhibitors N^G-monomethyl-L-arginine (L-NMMA) and L-N^5(1-iminoethyl)ornithine (L-NIO), the guanylate cyclase inhibitor 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ) and Triton X-100, a detergent that damages the endocardial endothelium, all increased stroke volume (V_S) and stroke work (W_S). In contrast, the endogenous NOS substrate L-arginine, tested before and after treatment with haemoglobin, the NO donor 3-morpholinosydnonimine, tested with and without the superoxide scavenger superoxide dismutase, and the stable cGMP analogue 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP) decreased V_S and W_S. Acetylcholine chloride produced a biphasic effect. At nanomolar concentrations, in 34 % of the preparations, it induced a NO–cGMP-dependent positive inotropism that required the integrity of the endocardial endothelium. Pretreatment with Triton X-100 or with NO–cGMP pathway inhibitors (L-NMMA, L-NIO, N^G-nitro-L-arginine methyl ester, Methylene Blue and ODQ) abolished the positive effect of acetylcholine. In contrast, at micromolar concentrations, acetylcholine produced a negative effect that involved neither the endocardial endothelium nor the NO–cGMP pathway. Pre-treatment with L-arginine (10^{-6} mol l^{-1}) was without effect, whereas L-NIO (10^{-5} mol l^{-1}) significantly reduced the Frank–Starling response. Taken together, these three experimental approaches provide evidence that NO modulates cardiac performance in the eel heart.

Key words: nitric oxide, cardiac performance, fish, Frank–Starling response, eel, *Anguilla anguilla.*

Introduction

Cardiac function is modulated by intrinsic (Frank–Starling mechanism, frequency/force relationship, shear stress, etc.) and extrinsic (neural and humoral) mechanisms. In mammals, both mechanisms are modulated by nitric oxide (NO) synthesized and released by endothelial, myocardial and interstitial cells, coronary vessels and myocardial neurons (Balligand, 2000). Nitric oxide alters diastolic properties (Smith et al., 1991), modulates the Frank–Starling response (Prendergast et al., 1997), the force/frequency relationship (Kaye et al., 1996) and the β-adrenergic (Hare et al., 1995) and cholinergic (Balligand, 2000) inotropic effects and induces positive inotropism (Méry et al., 1994). In both mammalian and amphibian hearts, NO targets guanylyl cyclase, an enzyme that is involved in the production of cGMP (Fischmeister and Méry, 1996). NO also interacts with haem proteins, protein thiols and iron/non-haem complexes and can react with superoxide anions to form peroxynitrite (Beckman and Koppenol, 1996).

Fish hearts are very sensitive to both intrinsic regulation (Frank–Starling response) and humoral control. In fact, perfused teleost hearts are much more responsive to filling pressure than perfused mammalian hearts. Indeed, in many mammalian hearts, input pressures must be 10 times higher than in teleost hearts to evoke maximal stroke volume (Farrell and Jones, 1992). For example, the eel heart displays the typical Frank–Starling response (maximum cardiac output 22.3±1.4 ml min^{-1} kg^{-1} body mass, body mass 2.7–6.3, at an input pressure of 378.7±43 Pa; means ± S.E.M., N=9) (Davie et al., 1992). This remarkable mechanical flexibility of the eel heart could have evolved in response to the variety of haemodynamic challenges experienced by the animal during its life cycle. In teleosts, the heart, innervated by a vagosympathetic trunk made up of cholinergic and adrenergic fibres (Laurent et al., 1983), is exposed to such humoral agents as catecholamines released by extracardiac and cardiac

chromaffin cells (Farrell and Jones, 1992; Nilsson and Holmgren, 1992). These substances could represent important chemical stimuli for the endocardial endothelium, which lines the extensive surface of the trabeculated fish ventricle. Compared with the compact type of ventricular myoarchitecture of homeotherm hearts, fish and amphibian hearts display a higher ratio of cavity surface area to ventricular volume, with a correspondingly greater mass of endocardial endothelium (Sys et al., 1997). The endocardial endothelium is a relevant source of NO (Smith et al., 1991); consequently, endocardial endothelium/nitrergic modulation of cardiac performance may be important in the teleost heart, as it is in the frog heart (Sys et al., 1997; Gattuso et al., 1999).

The purpose of this study was to investigate the putative role of NO in modulating eel cardiac performance under basal, cholinergic stimulation and loading (i.e. Frank–Starling response) conditions. To explore the specific autocrine role of the endocardial endothelium as a source of NO without the confounding effects of the vascular endothelium, we used juvenile eels in which the compact ventricular layer and the coronary circulation are poorly developed. Some preliminary results of this study have appeared in abstract form (Imbrogno et al., 2000).

Materials and methods

Isolated and perfused working heart preparation

We used specimens of both sexes of the European eel (*Anguilla anguilla* L.), weighing 98±3.23 g (mean ± S.E.M., N=292). Fish were provided by a local hatchery and kept at room temperature (18–20 °C) without feeding for 5–7 days. Experiments were performed between October and June. Each eel was anaesthetized in benzocaine (0.2 g l^{-1}) for 15 min. The animals were opened ventrally behind the pectoral fins. The hearts were removed without the pericardium and cannulated. Isolation time was 15–20 min. The cannulated heart was transferred to a perfusion chamber filled with Ringer's solution and connected with a perfusion apparatus (as described by Tota et al., 1991). The heart received Ringer's solution from an input reservoir and pumped against an afterload pressure given by the height of an output reservoir. The Ringer's solution contained the following (in g l^{-1}): NaCl, 6.68; KCl, 0.15; KH_2PO_4, 0.05; $MgSO_4$, 0.35; $(NH_4)_2SO_4$, 0.05; $CaCl_2$, 0.14; glucose, 1; Na_2HPO_4, 0.227; pH was adjusted to 7.7–7.9 by adding $NaHCO_3$ (approximately 1 g l^{-1}). The Ringer's solution was equilibrated with a mixture of O_2:CO_2 at 99.5:0.5 % (Davie et al., 1992). Experiments were carried out at room temperature (18–21 °C). Hearts were stimulated with a Grass S44 stimulator (frequency identical to that of control, non-paced hearts; pulse width fixed at 0.1 ms; voltage 1.2±0.1 V; means ± S.E.M.).

Measurements and calculations

The hearts were stabilized at the basal conditions (see below) for 15–20 min before being treated with drugs. Pressure was measured through T-tubes placed immediately before the input cannula and after the output cannula and connected to two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA, USA) in conjunction with a Unirecord 7050 (Ugo Basile, Comerio, Italy). Pressure measurements (input and output) were expressed in kPa and corrected for cannula resistance. Heart rate was calculated from pressure recordings. Cardiac output was collected over a period of 1 min and weighed; values were corrected for fluid density and are expressed as volumes. The afterload (mean aortic pressure) was calculated as two-thirds diastolic pressure plus one-third maximum pressure. Stroke volume (V_S; ml kg^{-1}; cardiac output/heart rate) was used as a measure of ventricular performance; changes in V_S were considered to be inotropic effects. Cardiac output and V_S were normalized per kilogram of wet body mass. Ventricular stroke work [W_S; mJ g^{-1}; (afterload–preload)×stroke volume/ventricle mass] served as an index of systolic functionality.

Experimental protocols

Basal conditions

Isolated perfused hearts were allowed to equilibrate to conditions simulating an *in vivo* resting state for up to 15–20 min. In all experiments, the control conditions were a mean output pressure of approximately 3.00 kPa, with cardiac output set to 10 ml min^{-1} kg^{-1} body mass by appropriately adjusting the filling pressure. These values are within the physiological range (Davie et al., 1992). The heart generated its own rhythm. Cardiac variables were measured simultaneously during experiments. To analyse the inotropic effects distinct from the chronotropic actions of substances, the preparations were electrically paced. Hearts that did not stabilize within 20 min of perfusion were discarded.

Time-course experiments

To assess the endurance of the preparation and to detect the onset of the hypodynamic state, the cardiac performance variables under basal conditions were measured every 10 min of perfusion for approximately 160 min.

Drug application

After the 15 min control period, the treated hearts were perfused for 20 min with Ringer's solution enriched with acetylcholine (ACh), L-arginine, L-arginine plus haemoglobin, 3-morpholinosydnonimine (SIN-1), SIN-1 plus superoxide dismutase (SOD), 3′,5′-cyclic monophosphate (8-Br-cGMP), N^G-monomethyl-L-arginine (L-NMMA), L-N^5(1-iminoethyl)ornithine (L-NIO) and Triton X-100. Each heart was tested for one concentration of the drug. The ACh-stimulated hearts tested for other drugs were perfused a second time with the medicated Ringer, which contained ACh plus an inhibitor [atropine, pirenzepine, AF-DX 116, L-NMMA, N^G-nitro-L-arginine methyl ester (L-NAME), L-NIO, Methylene Blue, 1H-[1,2,4]oxadiazole-[4,3-a]quinoxalin-1-one (ODQ), Triton X-100], after which performance variables were measured.

For Triton X-100 experiments, a needle was inserted into the posterior ventral region of the ventricular wall. Ten to fifteen

minutes after the onset of perfusion, when the heart had stabilized at basal conditions, 0.1 ml of 0.05 % Triton X-100 was injected through the needle into the ventricle to avoid damage to the atrium. At this concentration, the detergent is known not to affect the subjacent myocardium, as assessed by viability tracer and confocal microscopy (Sys et al., 1997). Control injections with 0.1 ml of Ringer's solution instead of Triton X-100 did not alter cardiac variables (data not shown), indicating that it was the detergent and not the perfusion manipulation that affected cardiac performance. Tests with Evans Blue solution demonstrated that the substance rapidly invaded the ventricle and was washed out after two or three beats; these tests also showed that no backflow into the atrium occurred during this procedure. Variables of cardiac performance were measured after 20 min of perfusion with the Ringer's solution. In the hearts pretreated with detergent that were to be tested for the effects of ACh, this 20 min period was followed by a 20 min perfusion with the ACh-enriched Ringer's solution, after which the performance variables were measured.

Frank–Starling response

To assess the interaction between NO and the Frank–Starling response, a Starling curve was generated (baseline condition). After baseline assessment, the input pressure was returned to the control condition and a second Starling curve (untreated time-control) was generated. These time-control curves were compared with Starling curves obtained in the presence of the endogenous substrate for NOS, L-arginine, or the nitric oxide synthase (NOS) inhibitor L-NIO.

Statistical analyses

The results are expressed as means ± S.E.M. Each heart received only one concentration of the drug. Because each heart acted as its own control, the statistical significance of differences was assessed using the paired Student's *t*-test ($P<0.05$). Percentage changes were evaluated as means ± S.E.M of percentage changes obtained from individual experiments. We used Student's *t*-tests on absolute values for within-group comparisons of the Starling curves; comparisons between groups were made using two-way analysis of variance (ANOVA). Significant differences from the time-control group were detected using Duncan's multiple-range test.

Drugs and chemicals

All solutions were prepared in double-distilled water (except ODQ, which was prepared in ethanol); dilutions were made in Ringer's solution just before use. Acetylcholine chloride, atropine sulphate salt, pirenzepine dihydrochloride, Triton X-100, L-NAME, L-NMMA, L-NIO, Methylene Blue, ODQ, 8-Br-cGMP, SIN-1 (used in a darkened perfusion apparatus to prevent degradation), L-arginine and haemoglobin were purchased from Sigma Chemical Company (St Louis, MO, USA). 11-({[2-(diethylamino)methyl]-1-piperidinyl}acethyl)-5,11-dihydro-6H-pyridol(2,3-b)(1,4)benzodiazepine-6-one (AF-DX 116) was a generous gift from Boehringer Ingelheim (Biberach, Germany).

Results

Isolated working heart preparations

The haemodynamic characteristics of the preparation compare well with the mechanical performance of the eel heart (Davie et al., 1992). Baseline haemodynamic variables are listed in Table 1. Typical time-course curves of heart rate and V_S indicate that the performance of the heart was stable for more than 2 h, after which the heart fell into a hypodynamic state characterized by a linear decrease in cardiac output, without significant changes in heart rate (Fig. 1B). On the basis of this endurance profile, all the following experiments were carried out within 2 h. Fig. 1A shows two typical pressure recording traces under control conditions (left) and after the addition of 10^{-6} mol l^{-1} ACh (right).

The perfused eel heart displayed a typical Frank–Starling curve (data not shown). Maximum cardiac output was 27±0.57 ml min^{-1} kg^{-1} body mass, and maximum stroke volume was 0.68±0.13 ml kg^{-1} body mass, obtained with an input pressure of 0.55±0.055 kPa (N=8).

Effects of NO on basal cardiac performance

To determine whether NO affects basal cardiac performance, the heart preparations were exposed to L-arginine (10^{-7} mol l^{-1}), the natural substrate for NOS. L-Arginine induced a significant decrease in V_S and W_S. These effects of L-arginine were due to NO because they were abolished by haemoglobin (10^{-6} mol l^{-1}), which inactivates NO (Moncada et al., 1991). A dose/response curve for SIN-1 (10^{-12} to 10^{-7} mol l^{-1}), an exogenous donor of NO, showed similar negative inotropic effects. Because SIN-1 releases NO and superoxide (Beckman and Koppenol, 1996), we tested its effects in the presence of superoxide dismutase (SOD) (10 units ml^{-1}). Pre-treatment with SOD did not modify the negative inotropism, which indicates that this effect was due to NO and not to the formation of peroxynitrite (Fig. 2). Pretreatment with the NOS inhibitors L-NMMA (10^{-5} mol l^{-1})

Table 1. *Performance variables under basal conditions of isolated eel heart preparations perfused with oxygen saturated Ringer's solution*

	Heart rate (beats min^{-1})	Filling pressure (kPa)	Output pressure (kPa)	Cardiac output (ml min^{-1} kg^{-1})	Stroke volume (ml kg^{-1})	Stroke work (mJ g^{-1})
Control	51.4±12.2	0.07±0.008	2.97±0.034	10.9±1.56	0.21±0.09	0.105±0.03

Results are expressed as mean values ± S.E.M. of 284 experiments.

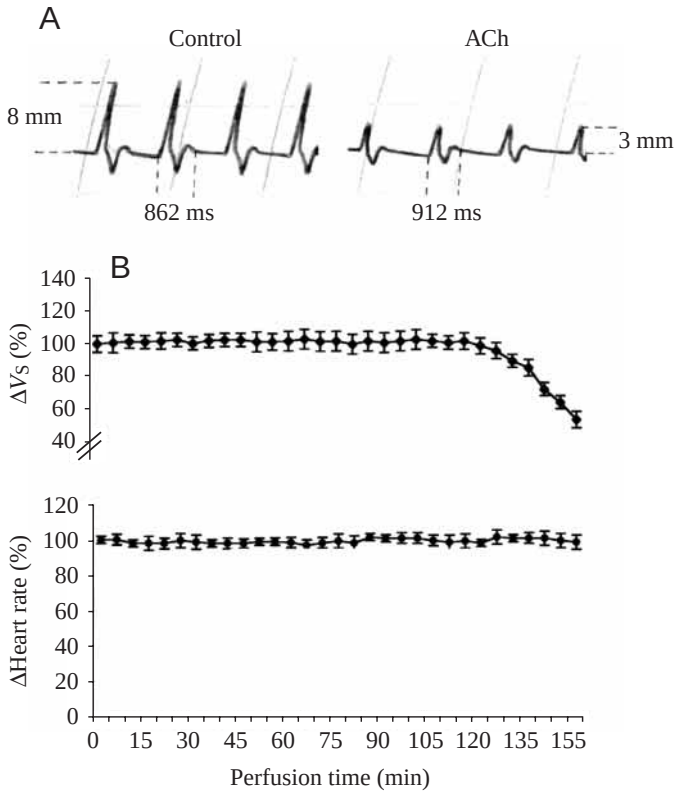

Fig. 1. (A) Two typical pressure recording traces under control conditions (left) and after the addition of 10^{-6} mol l^{-1} acetylcholine (ACh) (right). (B) Time course of stroke volume (V_S) and heart rate in control conditions (N=6 experiments). Data are expressed as a percentage of the value at 5 min of perfusion, i.e. after stabilization, and are means ± S.E.M.

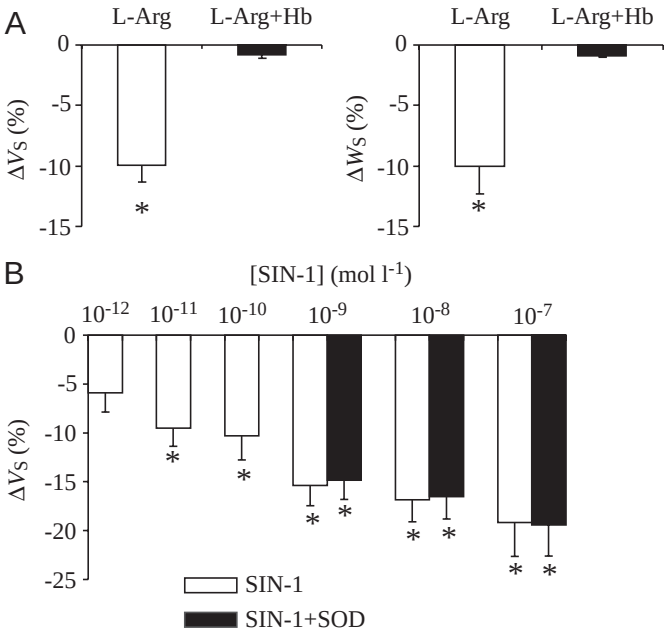

Fig. 2. (A) Effects of L-arginine (L-Arg; 10^{-7} mol l^{-1}) and haemoglobin (Hb; 10^{-6} mol l^{-1}) on stroke volume (V_S) and stroke work (W_S) in isolated and perfused paced eel hearts. Percentage changes were evaluated as means + S.E.M. of six experiments. (B) Dose/response curve for 3-morpholinosydnonimine (SIN-1) (from 10^{-12} to 10^{-7} mol l^{-1}) on stroke volume, in the presence and absence of superoxide dismutase (SOD; 10 units ml^{-1}), in isolated and perfused paced eel hearts. Percentage changes were evaluated as means + S.E.M. of four experiments for each concentration. *Significantly different from the control value (P<0.05). The W_S values for SIN-1 (10^{-12} to 10^{-7} mol l^{-1}) were –3.33±2 %, –9.77±2.21 %, –12.66±2.68 %, –13.85±2.33 %, –17.2±2.69 % and –19.44±3.3 %, respectively. The W_S values for SOD+SIN-1 (10^{-9} to 10^{-7} mol l^{-1}) were –14±2.5 %, –17±3 % and –19±2.7 %, respectively.

and L-NIO (10^{-5} mol l^{-1}) induced a positive inotropism (Fig. 3).

To assess whether the effects of NO were due to a cGMP-dependent mechanism, the preparations were exposed to a stable analogue of cGMP, 8-Br-cGMP at 10^{-9} to 10^{-6} mol l^{-1}. This treatment elicited negative inotropism (Fig. 4). In agreement with this finding, a specific inhibitor of guanylate cyclase, ODQ (10^{-5} mol l^{-1}), induced positive inotropism (Fig. 3).

We investigated the involvement of the endocardial endothelium in the regulation of eel cardiac function: Triton X-100 (0.05 %), a detergent that at this concentration damages the endocardial endothelium functionally but not structurally (see Sys, 1997), significantly increased V_S and W_S (Fig. 5).

Cholinergic stimulation: functional impairment of the endocardial endothelium and NO

Acetylcholine generally produces negative chronotropic and inotropic effects in the heart. In our study, in addition to the classic negative inotropism induced by ACh at 10^{-6} mol l^{-1}, at lower concentrations (10^{-11} and 10^{-10} mol l^{-1}) the cholinergic agonist produced positive inotropism in approximately 34 % of preparations over the whole year (Fig. 6A). The positive effect occurred mainly during spring (65 % of the preparations). Preliminary experiments on spontaneously beating preparations showed that ACh (10^{-12} to 10^{-6} mol l^{-1}) caused negative chronotropism, but the effect was significant only at 10^{-6} mol l^{-1} (data not shown). Repeated exposures demonstrated that ACh did not cause tachyphylaxis (data not shown).

The cholinergic effects of ACh were mediated by muscarinic receptors (mAChRs): atropine (an unspecific muscarinic antagonist, 10^{-6} mol l^{-1}) antagonized both the positive and the negative effects of ACh on stroke volume; pirenzepine (an M_1-specific antagonist) blocked the positive response at 10^{-8} mol l^{-1} and, only at a much higher concentration (10^{-5} mol l^{-1}), the negative response; AF-DX 116 (an M_2-specific antagonist) blocked the negative effect at 10^{-7} mol l^{-1} (Fig. 6B). To determine whether an intact endocardial endothelium is required for the cholinergic responses, hearts were pretreated with Triton X-100. This detergent blocked the positive cholinergic response, but did not modify the negative response (Fig. 7). Taken together, these results indicate that the positive inotropic response is mediated by M_1 muscarinic receptors, located largely on the endocardial endothelium,

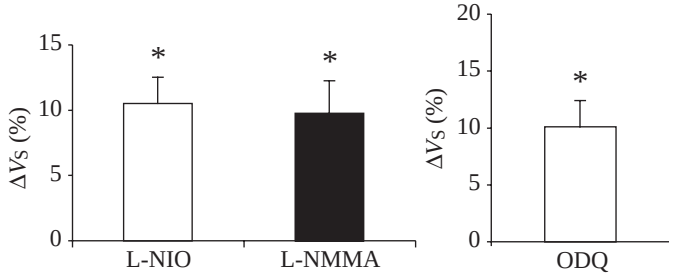

Fig. 3. Effects of *N*-iminoethyl-L-ornithine (L-NIO; 10^{-5} mol l^{-1}), N^G-monomethyl-L-arginine (L-NMMA; 10^{-5} mol l^{-1}) and 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ; 10^{-5} mol l^{-1}) on stroke volume (V_S) in isolated and perfused paced eel hearts. Percentage changes were evaluated as means + S.E.M. of four experiments for L-NIO and L-NMMA and six experiments for ODQ. *Significantly different from the control value (P<0.05).

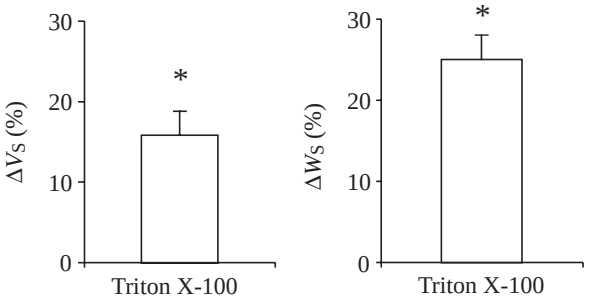

Fig. 5. Effects of Triton X-100 (0.05 %) injections on stroke volume (V_S) and stroke work (W_S) in isolated and perfused paced eel hearts. Percentage changes were evaluated as means + S.E.M. of four experiments. *Significantly different from the control value (P<0.05).

while the negative response is mediated by M_2 muscarinic receptors, located largely in the myocardiocytes.

We tested whether the cholinergic responses of the eel heart involved a nitrergic mechanism. Pretreatment with the NOS inhibitors L-NMMA (10^{-5} mol l^{-1}), L-NAME (10^{-4} mol l^{-1}) and L-NIO (10^{-5} mol l^{-1}) and the soluble guanylate cyclase blockers Methylene Blue (10^{-6} mol l^{-1}) and ODQ (10^{-5} mol l^{-1}) abolished the positive inotropic response to ACh, but did not affect the negative response (Fig. 8). These inhibitors did not affect chronotropism in spontaneously beating preparations (data not shown). Therefore, the positive inotropic action of ACh involves a NO–cGMP signal-transduction mechanism, which in turn requires an intact endocardial endothelium. In contrast, neither a NO–cGMP mechanism nor the endocardial endothelium appears to be involved in the negative cholinergic response.

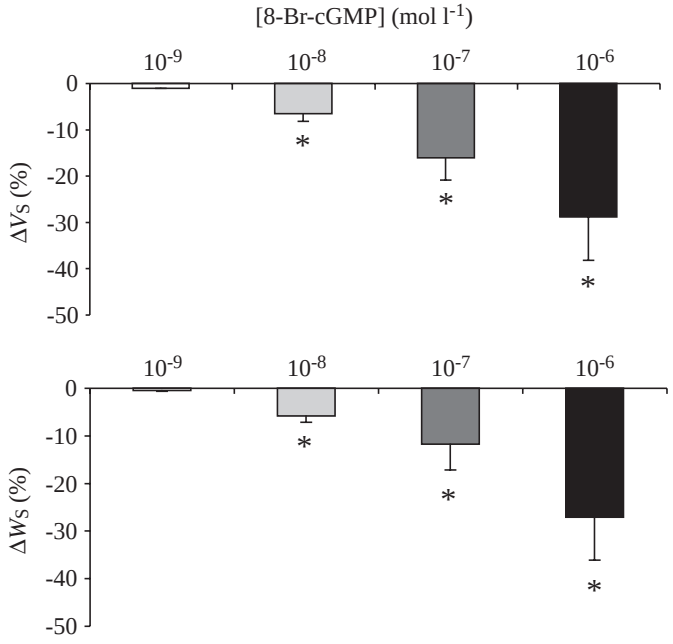

Fig. 4. Dose–response curve for 8-Br-cGMP (from 10^{-9} mol l^{-1} to 10^{-6} mol l^{-1}) on stroke volume (V_S) and stroke work (W_S) in isolated and perfused paced eel hearts. Percentage changes were evaluated as means + S.E.M. of four experiments for each concentration. *Significantly different from the control value (P<0.05).

Nitric oxide and the Frank–Starling response

To ascertain the effect of NO on the Frank–Starling response of the isolated and perfused eel heart, we studied this response before and after treatment with L-arginine (10^{-6} mol l^{-1}) and L-NIO (10^{-5} mol l^{-1}). Pre-treatment with L-arginine was without effect, whereas L-NIO significantly reduced the Frank–Starling response (Fig. 9). To separate the time factor (i.e. the 'memory' of the heart) of loading stimulation, we generated baseline and time-control curves. ANOVA showed that the curves were identical within experimental error.

Discussion

Isolated working heart preparations

The response to loading stimuli of the *in vitro* heart preparation used in this study mimics the haemodynamic response of the *in vivo* eel heart. Since mechanical stresses induce the release of autocrine/paracrine factors, including NO, that affect cardiac performance (Brutsaert and Andries, 1992; Pinsky et al., 1997; Kanay et al., 1995), it was important to ensure that the isolated, perfused working heart generated physiologically comparable values of output pressure, cardiac output and ventricular work.

Basal nitrergic tone

The results obtained in this study with the endogenous (L-arginine) and exogenous NO donors, the NOS and guanylate cyclase inhibitors and 8-Br-cGMP indicate that, in the eel heart, an active NO–cGMP pathway exerts a mild but significant negative inotropic effect on basal (i.e. unstimulated) cardiac performance. The continuous generation of relatively low basal levels (nanomolar concentrations) of NO, mainly associated with endothelial NOS (cNOS) activity, is considered critical for the functional integrity of the

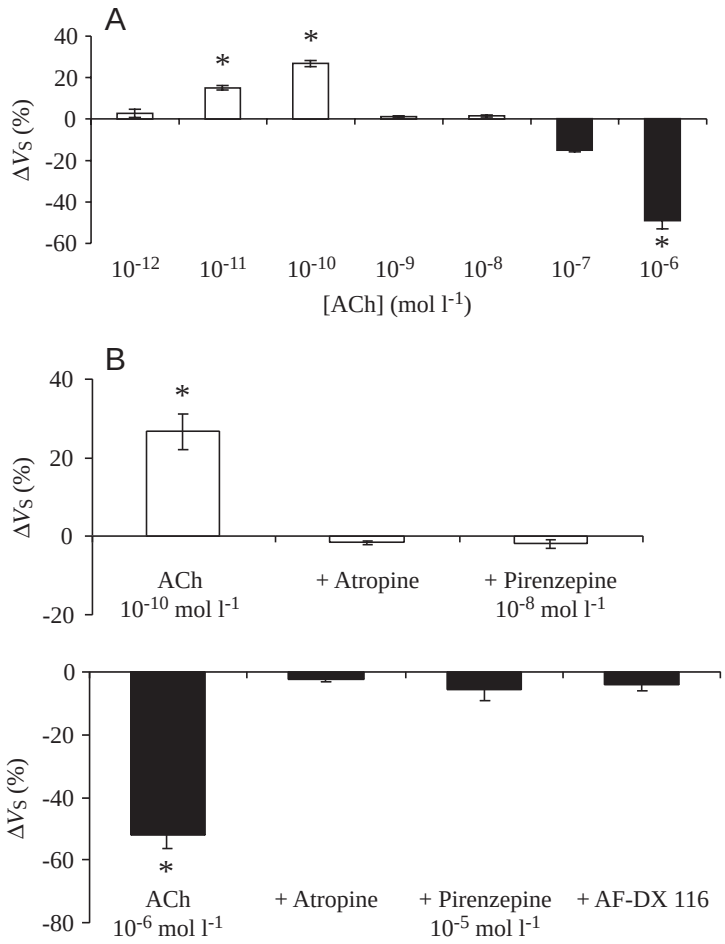

Fig. 6. (A) Dose/response curve for acetylcholine (ACh; from 10^{-12} to 10^{-6} mol l^{-1}) on stroke volume (V_S) in isolated and perfused paced eel hearts. Percentage changes were evaluated as means + S.E.M. of 17–18 experiments for each concentration. The corresponding W_S values were: 4.9±1.5 %, 17.9±2.3 %, 16.67±1.1 %, 2.94±1.1 %, 2.78±1 %, –12.5±1 % and –35.29±1 %. (B) Effects of ACh (10^{-10} and 10^{-6} mol l^{-1}) after pretreatment with atropine (10^{-6} mol l^{-1}), pirenzepine (10^{-8} mol l^{-1} and 10^{-5} mol l^{-1}) and AF-DX 116 (10^{-7} mol l^{-1}) on stroke volume in isolated and perfused paced eel hearts. Percentage changes were evaluated as means + S.E.M. of four experiments for each drug. *Significantly different from the control value (P<0.05).

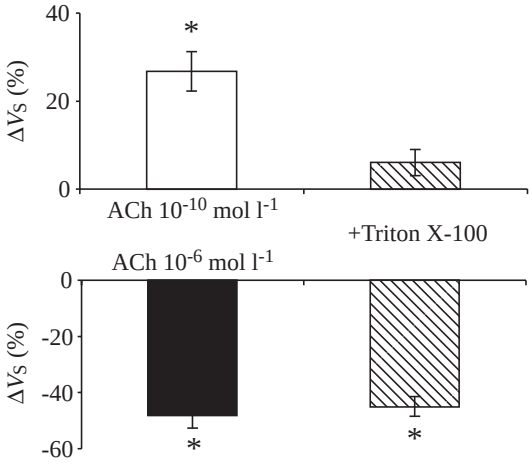

Fig. 7. Effects of acetylcholine (ACh; 10^{-10} and 10^{-6} mol l^{-1}) after pretreatment with Triton X-100 (0.05 %) on stroke volume (V_S) in isolated and perfused paced eel hearts. Percentage changes were evaluated as means ± S.E.M. of four experiments. *Significantly different from the control value (P<0.05).

mammalian heart (Moncada et al., 1991). The negative inotropic effect of increased NO levels mediated by cGMP has been reported in mammals (Grocott-Mason et al., 1994; Balligand, 2000). In the frog, increased cGMP levels in ventricular muscle depressed contractility (Singh and Flitney, 1981).

In the present study, all the interventions elicited inotropic effects of similar magnitude (changes of between approximately 10 and 15 %). Interestingly, we obtained results comparable with those of previous studies: the same drug concentrations induced effects of a similar magnitude in *Rana esculenta*, which is also characterized by a fully trabeculated avascular ventricle (Sys et al., 1997). In both preparations, loading conditions and heart rate can be controlled, while the vascular endothelium component is either absent (frog) or excluded (eel). This is an important point. In fact, the conclusions that can be drawn very much depend on the experimental preparation and techniques used to analyse whether, and to what extent, basal NO production influences myocardial performance in the absence of loading and of agonist stimulation. This may help explain some of the conflicting results obtained in previous studies of mammals. For example, stimulation of cNOS in intact hearts elicits lusitropic (i.e. relaxant) effects that are not observed in similarly stimulated ventricular papillary muscle preparations (Pinsky et al., 1997). Physiological changes in ventricular loading conditions affect cardiac NO synthesis, the mechanical stimulation being transduced into NO production in the heart *via* the endocardial endothelium (Pinsky et al., 1997). Endothelial shear stress increases NO concentration by stimulating constitutive NOS (Kanay et al., 1995) whereas, in both resting and contracting skeletal muscles, physiological levels of stretch significantly increase caveolae neck diameter and membrane surface area (Dulhunty and Franzini-Armstrong, 1975).

As in the frog heart (Sys et al., 1997), experiments with Triton X-100 suggest that, in the eel ventricle, the endocardial endothelium is an important source of NO because functional impairment of the endocardial endothelium abolished the negative nitrergic inotropism. The possible mechanism whereby Triton X-100 depresses basal release of NO from the endocardial endothelium has been described elsewhere (Sys et al., 1997).

Cholinergic stimulation

In the heart, cholinergic stimuli are mediated by several mAChR subtypes whose relative amounts vary among species

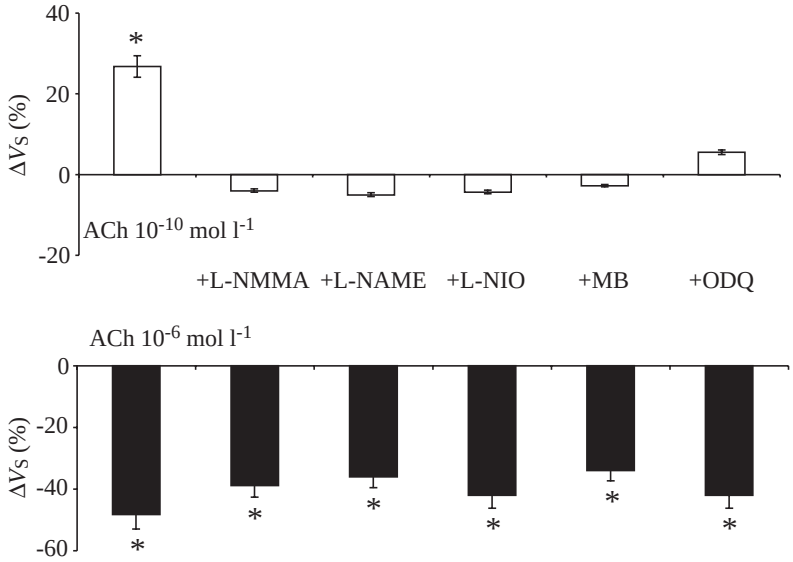

Fig. 8. Effects of acetylcholine (ACh; 10^{-10} and 10^{-6} mol l^{-1}) after pretreatment with N^G-monomethyl-L-arginine (L-NMMA; 10^{-5} mol l^{-1}), L-NAME (10^{-4} mol l^{-1}), *N*-iminoethyl-L-ornithine (L-NIO; 10^{-5} mol l^{-1}), Methylene Blue (MB; 10^{-6} mol l^{-1}) and 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ; 10^{-5} mol l^{-1}) on stroke volume (V_S) in isolated and perfused paced eel hearts. Percentage changes were evaluated as means ± S.E.M. of five experiments for each drug. *Significantly different from the control value (P<0.05).

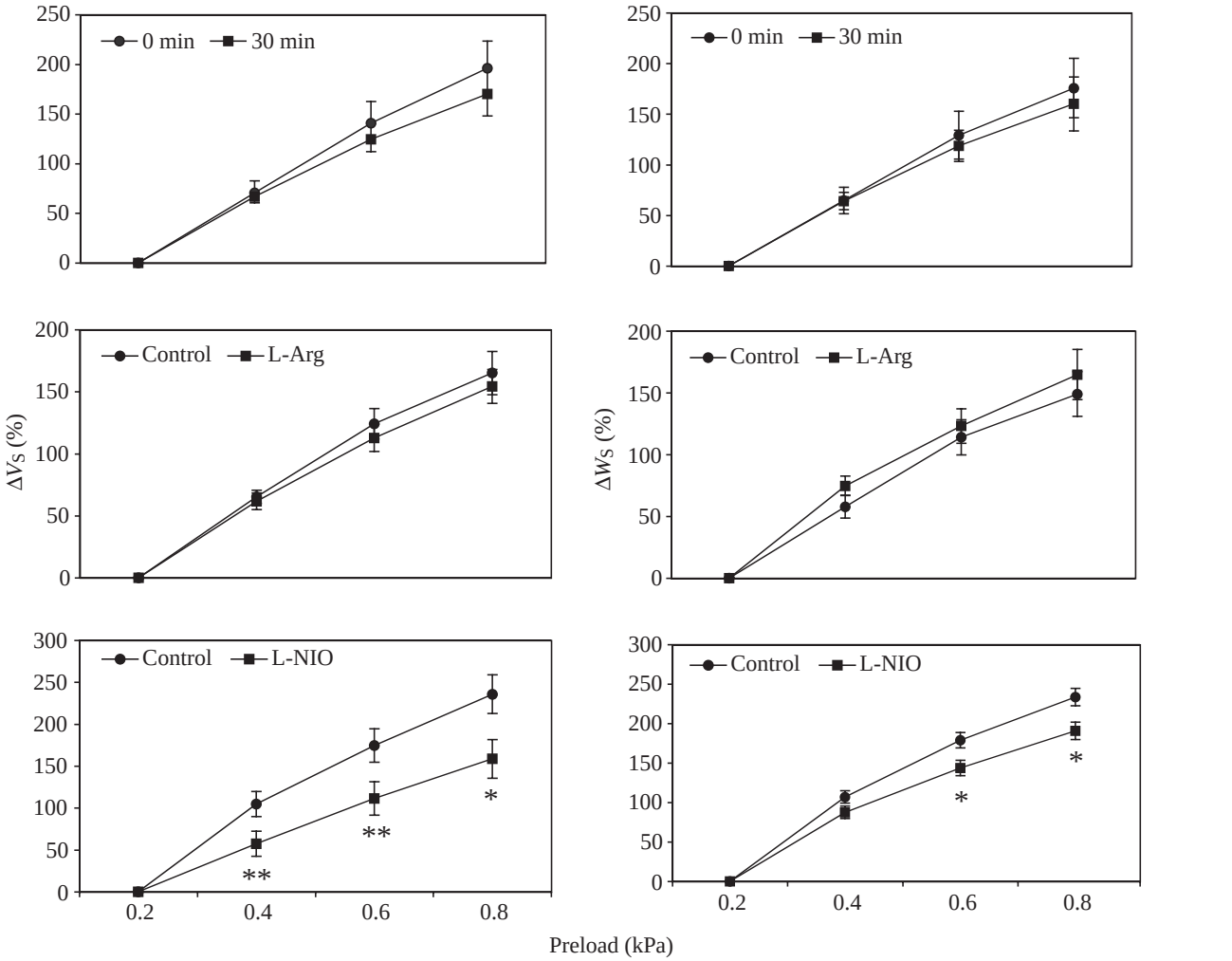

Fig. 9. Effects of preload on stroke volume (V_S) and stroke work (W_S) in control conditions and after pretreatment with *N*-iminoethyl-L-ornithine (L-NIO; 10^{-5} mol l^{-1}) and L-arginine (L-Arg; 10^{-6} mol l^{-1}) in isolated and perfused paced eel hearts. Percentage changes were evaluated as means ± S.E.M. of four experiments for each group. A paired Student's *t*-test was used for comparisons within groups; a two-way ANOVA was used for comparison between groups. Asterisks mark values that are significantly different from the control value (*P<0.05; **P<0.01). The top two panels show the baseline control (0 min) and the untreated time-control (30 min) (see Materials and methods).

and tissues. The M_2 and M_4 subtypes, preferentially located on the myocardiocytes and principally coupled to adenylate cyclase inhibition, reduce intracellular cAMP levels and decrease the L-type Ca^{2+} current, thereby eliciting negative cholinergic chronotropic and inotropic effects (for references, see Hove-Madsen et al., 1996; Gattuso et al., 1999). The M_1, M_3 and M_5 subtypes, which are located largely on the vascular endothelial and endocardial endothelium cells, are functionally coupled to mobilization of intracellular Ca^{2+} *via* phospholipase C and also phospholipase A_2 and phospholipase D, thereby mediating positive cholinergic inotropism (Brodde and Michel, 1999). Constitutive NOS activity linked to the muscarinic cholinergic signal-transduction cascade has been documented in ventricular myocytes and in the endocardium of the heart of several mammalian species (for references, see Balligand, 2000). However, it is debatable whether a NO–cGMP signalling pathway is essential in mediating cholinergic signalling in cardiac preparations and cardiomyocytes. Indeed, controversial results have been obtained in mice lacking endothelial NOS (Han et al., 1998; Vandecasteele et al., 1999).

In agreement with data obtained from amphibian hearts (Gattuso et al., 1999) and in *Anguilla japonica* (Chan and Chow, 1976), exogenous ACh had a significant biphasic inotropic effect on heart preparations from *Anguilla anguilla*. While in the frog heart both cholinergic inotropic effects are mediated by a NO–cGMP signal-transduction mechanism and require the functional integrity of the endocardial endothelium (Gattuso et al., 1999), the present study shows that in the heart of *Anguilla anguilla* the endocardial endothelium and the NO–cGMP mechanism appear to be involved only in the positive cholinergic response. In fact, Triton X-100 and drugs that block various steps of the NO–cGMP signalling pathway, L-NMMA (10^{-5} mol l^{-1}), L-NAME (10^{-4} mol l^{-1}), L-NIO (10^{-5} mol l^{-1}), Methylene Blue (10^{-6} mol l^{-1}) and ODQ (10^{-5} mol l^{-1}), abolished the positive effects of ACh but did not affect the negative effects.

Seasonal changes in cardiac reactivity to catecholamines have been reported in *Anguilla anguilla* (Peyraud-Waitzenegger et al., 1980). Our finding that the positive cholinergic response is observed mostly in the spring supports the idea that seasonal factors can fine-tune the sensitivity of the eel heart. Acylated NOS has been detected in endothelial cell caveolae, which are the location for many proteins involved in signal-transduction cascades, including tissue factors, platelet-derived growth factor receptors, muscarinic cholinergic receptors, protein kinase C, G-proteins, G-protein-linked receptors, Ca^{2+} channels and the plasmalemmal Ca^{2+}-ATPase (Balligand, 2000). It is reasonable to assume that the co-localization of NOS and such other proteins as muscarinic cholinergic receptors in the restricted space of the caveolae may provide a temporally and spatially 'delimited' pathway for signal transduction.

Nitric oxide and the Frank–Starling response

It is well established that the Frank–Starling response contributes to the regulation of cardiac output in the heart both *in vivo* and *in vitro* by interacting with such mechanisms as heart rate, neurohumoral modulation and coronary flow (for mammals, see Lakatta, 1992; for fish, see Farrell and Jones, 1992). The underlying mechanism involves a length-dependent change in myofilament responsiveness to Ca^{2+} (Lakatta, 1992; Crozatier, 1996). Studies with mammalian heart preparations suggest that cardiac NO influences the Frank–Starling response (Prendergast et al., 1997), probably by acting on either systolic or diastolic functions (Grocott-Mason et al., 1994). For example, a NO-induced alteration in ventricular diastolic properties was postulated consequent to the finding that the exogenous NO donor sodium nitroprusside and substance P (an agonist for NO release from endothelial cells) caused a shift in the position of the pressure/volume curve compatible with reduced diastolic stiffness (Paulus et al., 1994). This change could be achieved through Ca^{2+}-dependent or Ca^{2+}-independent mechanisms (Shah, 1996). In isolated rat cardiac myocytes, the cGMP analogue 8-Br-cGMP increases diastolic myocyte length and reduces myofilament responses to Ca^{2+} (Shah et al., 1994). Alternatively, as documented in studies on the frog heart (Méry et al., 1994), one may envisage mechanisms involving a reduction in intracellular Ca^{2+} concentration such as that secondary to stimulation of cGMP-dependent cAMP phosphodiesterases.

Our data show that in the fish heart there is relevant nitrergic modulation of the Frank–Starling relationship. In fact, while pre-treatment with L-arginine did not significantly influence the preload-induced increases in stroke volume, L-NIO significantly reduced them.

Although L-arginine exerts a negative inotropic effect on basal cardiac performance, it has no effect on the Frank–Starling response. L-arginine availability could be limiting in the presence of NO synthesis agonists, but not under basal conditions (Pollock et al., 1991). Alternatively, differences in the effect of exogenous L-arginine on basal and stimulated NO synthesis are suggested by results obtained with the isolated perfused rabbit heart (Smith et al., 1992) and in neutrophils and platelets (Radomski et al., 1990). In contrast, the dramatic effect elicited by L-NIO stresses the importance of basal nitrergic modulatory tone for the Frank–Starling response in the eel heart. As suggested for the mammalian heart (Brutsaert and Andries, 1992), the endocardial endothelium could act as an important cardiac mechanosensor of luminal forces in the fish heart.

In conclusion, this study shows that NO modulates ventricular performance in the working eel heart *in vitro*, and this is the first such demonstration in fish. Under unstimulated conditions, a basal nitrergic tone exerts a mild, but significant, negative inotropic effect *via* a cGMP-dependent mechanism. This nitrergic tone requires the functional integrity of the endocardial endothelium, which may be a major source of NO. The endocardial endothelium mediates, also *via* a NO–cGMP pathway, the positive inotropism elicited by luminal cholinergic stimuli (picomolar to nanomolar range), hence participating in the ventricular fine-tuning of the molecular signalling cascade downstream from the stimulation of the

cardiac muscarinic receptors. Of major interest is the finding that basal release of endogenous NO significantly influences the Frank–Starling response. The Frank–Starling response *in vivo* contributes to the increase in cardiac output associated with exercise (Farrell and Jones, 1992). Since fish have a less marked chronotropic response to exercise than mammals, this response is of greater importance in fish. Taken together, these results suggest that NO plays a major role in the regulation of ventricular performance in the fish heart.

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